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| (21) International Application Number: PCT/US91/02580 (22) International Filing Date: 15 April 1991 (15.04.91) (30) Priority data: 510,178 17 April 1990 (17.04.90) US (71) Applicant: ANALYTICAL CONTROL SYSTEMS, INC. [US/US]; Seven Technology Drive, Noblesville, IN 46060 (US). (72) Inventor: SPECK, Roy, E. ; 3636 Paddington Circle, Indianapolis, IN 46268 (US). (74) Agents: McNETT, John, C. et al.; Woodard, Emhardt, Naughton, Moriarty & McNett, Bank One Center/Tower, 111 Monument Circle, Suite 3700, Indianapolis, IN 46204 (US). | | (81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH (European patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent). Published <i>With international search report.</i> |
| (54) Title: COAGULATION ASSAYS AND REAGENTS (57) Abstract Novel assays and reagents for determining coagulative properties of blood or plasma are disclosed, as are agents and methods for stemming bleeding. Novel ellagic acid-based activators consist essentially of aqueous solutions of ellagic acid, phenol and suitable metal ions and give defined values for a novel platelet assay also disclosed. Inventive coagulation reagents including propyl gallate or tannin are also disclosed, as are aPTT reagents having sensitivity to heparin and Factor deficiencies far superior to prior aPTT reagents. Disclosed heparin-sensitive reagents include dextran sulfate. Also disclosed is applicant's discovery that reagents including hydroxy-substituted compounds and metal ions, heretofore known to activate Hagemann Factor, also cause platelets to release Platelet Factor 3, and a series of platelet-sensitive tests found to be enabled by this discovery. These platelet-sensitive tests include assays for platelet activity, systemic lupus, platelet reserve, which can signify type II lipoprotein defects, and for Platelet Factor 4. Disclosed methods and materials for stemming bleeding center around applicant's discovery that bleeding from bleeding sites can be stopped up to six time faster by applying to the site a hemostatic agent including effective amounts of hydroxy-substituted aromatic compound and metal ion. | | |

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COAGULATION ASSAYS AND REAGENTS

BACKGROUND OF THE INVENTIONS

The present inventions relate generally to medical diagnosis and treatment, and more particularly to assays and reagents for determining coagulative properties of blood or plasma, and to agents and methods for promoting clotting.

By way of background, the ability of animals to selectively form blood clots in areas of trauma is a vital function. Failure of the blood to clot, of course, can lead to severe hemorrhage and in some instances eventual fatality. On the other hand, uncontrolled clotting or coagulation of the blood within vessels can also lead to serious complications. In light of these and other complications related to blood clotting, there has naturally been a great desire to develop tests which can be used to measure clotting tendencies and to determine the cause of any abnormalities, as well as methods and materials for treating bleeding sites.

A number of tests have been developed to monitor or determine the causes of abnormal blood clotting tendencies. Although these known tests have in some instances proven acceptable to some extent, especially in light of the desire to improve the human condition, there is a continuing need for even more sensitive blood clotting tests which give consistent and reproducible results. Additionally, improvements need to be made in the reagents for these prior art tests, which are commonly turbid or include undesirable particulate matter, and which have solid, non-soluble activating species, which fact is borne out by filtering these prior art reagents and noting substantial if not complete loss of coagulation activating ability. Further, there is an ever-present need for new assays for accurately

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monitoring or determining clotting conditions for which there are no known accurate tests.

For example, one general coagulation test procedure which has been developed is the activated partial thromboplastin time (also commonly referred to as the aPTT). Early on, a typical aPTT test was conducted by incubating a citrated plasma sample in contact with a solid material, such as glass, celite or kaolin, known to activate Factor XII (Hagemann Factor). Then, Ca^{2+} ion and a platelet substitute (i.e. a phospholipid such as a cephalin derived from brain tissue or soy bean) was added to the sample, and the time necessary for the sample to clot was measured. More recently, commercial aPTT reagents have been developed which include the platelet substitute and a chemical known to activate Hagemann Factor, such as ellagic acid. For example, commercial aPTT reagents are available from Dade Division of Baxter Travenol, of Miami Florida, Ortho Diagnostics of Raritan, New Jersey, and Nyegaard A.S. of Norway. In a typical use, these commercial aPTT reagents are added to a citrated plasma sample which is then incubated for a period of time (commonly about 5 minutes) for activation to occur. Ca^{2+} is then added to the sample, often in the form of CaCl_2 , and the time necessary for clotting is measured. However, these aPTT reagents have proven to be unsatisfactory in many aspects because they are not sensitive enough to heparin, Factor deficiencies, or other causes of abnormal clotting tendencies. Also, these commercial reagents give sporadic and unreproducible results in some instances, problems which some have attributed to the presence of particulate or otherwise non-dissolved matter in the reagents. Also, the activator in these commercial reagents not in solution, a fact which is confirmed by filtering the reagents and noting a substantial or complete loss of activating behavior.

Another generally known coagulation test procedure is the

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Activated Whole Blood Coagulation Time (AWBCT). Typical known AWBCT tests are performed by placing a whole blood specimen in a test tube containing solid particulate material such as celite for activation of Hagemann Factor.

- 5 Thereafter, the sample is heated and agitated, and the time necessary for the sample to clot is measured. As with the known aPTT tests, however, these prior art AWBCT tests often give unreliable and unreproducible results. This could result from activation with the solid particulate material.
- 10 Activation in this manner tends to be non-uniform and to interfere with normal coagulation mechanisms due to adsorption of Factors and other materials to the solid particles.

- In addition to the above-noted shortcomings of known tests and reagents, there has been a more wholesale failure in this area in providing blood clotting tests which are sensitive to the activity of blood platelets in the coagulation scheme. This is despite the existence of very numerous disorders and treatments which impact platelet activity.
- 15 20

- For example, one condition which is known to affect platelet activity is systemic lupus. This form of lupus is thought to be attributable to the presence of "lupus anticoagulant," which is an antiphospholipid antibody which inhibits the action of Platelet Factor 3 (PF3) in the coagulation mechanism. One test which has been suggested for use in detecting lupus anticoagulant is an ACT test performed on platelet poor plasma using kaolin to activate Hagemann Factor. T. Exner et al., British Journal of Haematology, 1978, 40, 143-51. While Exner et al. reported successfully detecting lupus anticoagulant using their method, it nonetheless involves using solid particulate material for activation which, as discussed above, can lead to decreased sensitivity and consistency from test to test.
- 25 30

- 35 The Tissue Thromboplastin Inhibition Procedure (TTI), M.

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Boxer et al., Arthritis Rheum. 19:1244 (1976); M.A. Schleider et al., Blood, 1976, 48, 499-509, and the Platelet Neutralization Procedure (PNP), D.A. Triplett et al., A.J.C.P., 79, No. 6, 678-82 (June 1983), have also been
5 suggested for use in detecting the presence of lupus anticoagulant. However, D.A. Triplett et al. demonstrated that the TTI procedure is not specific for lupus anticoagulants and thus does not provide a desirable test for detecting systemic lupus. Additionally, the PNP, while
10 having been demonstrated to be sufficiently sensitive for qualitative determination of lupus anticoagulant, see V. Dayton et al., Laboratory Medicine, January 1990, pp. 30-32, does not provide a test for qualitative and quantitative study of platelet activity, and relies upon the addition of
15 freeze-thawed platelets to neutralize the lupus anticoagulant.

In addition to detection of systemic lupus, there are also many other conditions which contribute to the need and desire for sensitive, reliable tests for platelet activity. For instance, it has long been known that aspirin (ASA)
20 inhibits the activity of platelets in the coagulation system by suppressing their release of PF3. This, in turn, can lead to extended coagulation times for blood and plasma of patients taking aspirin. Nonetheless, as is well known, aspirin has been widely used as a pain killer and
25 anti-inflammatory drug. Additionally, there has been a recent trend in medicine to prescribe a daily regimen of aspirin to reduce risk of heart attack. In fact, it has been estimated that over 20 million people in the U.S. presently take at least one aspirin a day for this reason. Further,
30 recent suggestions have been made that sufferers of migraine headaches can benefit from a daily regimen of aspirin, and this could lead to over two million additional persons in the U.S. on daily aspirin therapy. This extensive and rapidly growing use of aspirin, which has heretofore somewhat
35 recklessly proceeded without monitoring its effect on the

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patients' platelets, gives rise to an urgent need for sensitive tests which can be used to monitor aspirin therapy.

Another driving force for the development of good tests
5 for platelet activity is the existence of platelet function abnormalities in patients. As an example, it has been discovered that full-term pregnancy pre-eclamptic women often have prolonged bleeding times. This has been attributed to low platelet counts, and also in some instances is thought to
10 be due to abnormalities in platelets. See, J. Ramanathan et al., Anesthesiol., 1989, 71, 188-91.

Additionally, in the area of quality control, a recent article points up the need for a sensitive test which can be used to differentiate platelet concentrates which retain
15 functional integrity after storage from those which do not, and explains that there is presently no available method for accomplishing this. T. Hervig et al., Clin. Chem., 1990, 36, No. 1, pp. 28-31.

Moreover, the presence of antiphospholipid antibodies has
20 been associated with the occurrence of premature fetal death syndrome, see, for instance, D. A. Triplett, College of American Pathologists Today, July 1989, Vol. 3, No. 7 p. 61, thus giving rise to an additional group which would benefit from more sensitive and accurate tests for the presence of
25 antiphospholipid antibodies. Further, to date there are no tests known to applicant for the quantitative determination of Platelet Factor 4 (PF4). However, as is known, PF4 neutralizes heparin, and thus an accurate determination of a patient's PF4 level should be an important and routine part
30 of the application and monitoring of heparin therapy.

Another area to which this invention relates is hemostatic agents, which are commonly used to control bleeding from wounds or from vessels during surgery. As an example, collagen preparations have been used as topical
35 hemostatic agents. R.G. Mason et al., Haemostasis, 3, 31-45

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(1974). Mason et al. report that the collagen preparations apparently accelerate formation of fibrin primarily by alteration of platelets rather than by direct action on the soluble components of the intrinsic coagulation system. In this regard, product literature for AVITENE, a microfibrillar collagen hemostat (MCH) available from Alcon Laboratories, Inc. of Fort Worth, Texas, also states that contacting the MCH with a bleeding surface attracts platelets which adhere to the fibrils and undergo the release phenomenon to trigger aggregation of platelets into thrombi in the interstices of the fibrous mass. Physician's Desk Reference (1987) pp. 588-589. In addition to topical hemostats, it is also known to use hemostatic pastes to stem bleeding from arteries during major surgery such as heart surgery. Despite these known hemostatic agents, there still exists a continuing need and desire for improved hemostatic agents. The applicant's invention also contemplates an improved hemostatic agent to address this need.

As is evident from the foregoing, there exists a continuing need for new, as well as more sensitive, accurate and reliable coagulation assays which can be used to determine coagulative properties of blood or plasma. There also exist needs for improvements in reagents for clotting tests, and in materials and methods for treating bleeding. The applicant's inventions address these needs.

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SUMMARY OF THE INVENTIONS

A first preferred embodiment relates to a reagent for uniform activation of intrinsic coagulation which consists essentially of an aqueous solution of effective amounts of
5 ellagic acid, phenol, and a suitable metal ion. The ellagic acid and metal ion are fully solubilized, and consistent activated plasma clotting times of less than about 40 seconds can be achieved for normal platelet-rich plasma.

A second preferred embodiment relates to a method for
10 preparing a coagulation test reagent. This method comprises the steps of (a) preparing an aqueous solution by dissolving ellagic acid in water in the presence of a suitable base, (b) adding phenol to said aqueous solution, and (c) after said adding phenol, adding a suitable metal ion to said solution,
15 said phenol being added in amounts sufficient to inhibit formation of insoluble metal hydroxides of said suitable metal ion, whereby the overall reagent is a solution.

A third preferred embodiment relates to the applicant's discovery that tannin or propyl gallate, in combination with
20 a suitable metal ion, form an excellent activator of intrinsic coagulation. Accordingly, this embodiment involves a reagent for activation of intrinsic coagulation which contains a suitable metal ion and propyl gallate or tannin.

A fourth preferred embodiment relates to an aPTT reagent
25 which comprises freeze-dried platelet substitute reconstituted with an aqueous solution containing (i) a suitable hydroxy-substituted aromatic compound and (ii) a suitable metal ion. This reagent gives consistent activated partial thromboplastin times of less than about 40 seconds
30 for normal plasma, and, importantly, the overall reagent is a solution and has far superior sensitivity than prior aPTT reagents.

A fifth preferred embodiment relates to a coagulation test reagent kit. This kit comprises (i) a vial containing

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freeze-dried platelet substitute, and (ii) an aqueous solution comprising effective amounts of (i) a suitable hydroxy-substituted aromatic compound and (ii) a suitable metal ion. The hydroxy-substituted compound and suitable
5 metal ion are fully solubilized. Further, the solution and platelet substitute, when combined in controlled amounts, form an overall reagent which is also a solution and which gives consistent aPTT values of less than about 40 seconds for normal plasma and has superior sensitivity.

10 A sixth preferred embodiment relates to an improvement in a reagent for activation of intrinsic coagulation which includes a suitable metal ion and a suitable hydroxy-substituted aromatic compound. In accordance with the invention, this reagent also includes dextran sulfate in
15 effective amounts to increase sensitivity to heparin.

A seventh preferred embodiment relates to a platelet-sensitive method for assaying plasma for coagulation characteristics. This method includes the steps of (a) incubating at least one sample of the plasma in contact with
20 a reagent containing effective amounts of a suitable hydroxy-substituted aromatic compound and a suitable metal ion, (b) after the incubating, adding calcium to the plasma sample to initiate clotting, and (c) after the adding calcium, measuring the time necessary for the sample to clot,
25 the sample being at least essentially free from platelet substitute during clotting. In several preferred modes, this method provides assays for platelet activity, systemic lupus, PF4, platelet reserve, for signifying excessive circulating low-density lipoproteins, and platelet concentrate
30 integrity. These assays were discovered following the applicant's surprising finding that reagents containing suitable hydroxy-substituted aromatic compounds and metal ions, heretofore known to activate Hagemann Factor, also cause the release of Platelet Factor 3 from platelets. This
35 discovery is contrary to earlier reports in the field that

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ellagic acid does not alter platelet aggregation (platelet aggregation is generally considered a preliminary step to the release of PF3), A. Girolami et al., "Failure of Ellagic Acid to affect Platelet Aggregation in normal and Factor XII deficient Plasma," Blut, Band 31, Seite 107-112 (1975), or may even inhibit platelet aggregation. See J. Aznar et al., "Effect of Contact Factor (Factor XII + Factor XI) on Aggregation of Platelets," Haemostasis 3, 20-30 (1974).

An eighth preferred embodiment relates to a method for stemming bleeding, which comprises the step of (i) applying to a bleeding site an effective amount of a hemostatic agent including a suitable metal ion and a hydroxy-substituted aromatic compound.

The objects of the inventions are many. The inventions are intended to provide coagulation reagents and tests which are improved in sensitivity, uniformity of activation, and consistency in test results, and to provide sensitive and accurate assays for platelet activity and for conditions which affect measured platelet activity. Additional objects and advantages are apparent from the discussions both above and below.

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DESCRIPTION OF THE PREFERRED EMBODIMENTS

For the purposes of promoting an understanding of the principles of the inventions, reference will now be made to certain preferred embodiments and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the inventions is thereby intended, such alterations and further modifications, and such further applications of the principles of the inventions being contemplated as would normally occur to one skilled in the art to which the invention relates.

Improved Ellagic Acid Activator And Method For Preparation

As stated above, a first preferred embodiment relates to a reagent for uniform activation of intrinsic coagulation. This reagent consists essentially of an aqueous solution of ellagic acid, phenol, and suitable metal ion. The ellagic acid and suitable metal ion are fully solubilized, and consistent activated plasma clotting times of less than about 40 seconds can be achieved for normal platelet-rich plasma using the reagent.

As indicated, the reagent of this embodiment is a solution. Thus, the ellagic acid and suitable metal ion are fully solubilized, and the activity of the reagent, or at least a substantial percentage thereof, preferably about 85% or greater, and more preferably about 90-100%, is retained even after filtering through a 0.45 micron MILLIPORE filter. Accordingly, when herein a reagent is described as being a "solution", this means that a reagent's activity is substantially the same before and after filtering, such as through a 0.45 micron MILLIPORE filter. In this regard, it is preferred that the reagent, after such filtration, retain at least 85%, more preferably at least 90%, and most preferably levels approaching 100% of its original unfiltered activity. This retention of activity is evidenced by the

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similar respective clotting times for plasma obtained using the reagent before, and after filtration, as is further illustrated in specific Example 34 below.

Further, and also importantly, the reagent gives
5 consistent activated plasma clotting times (aPCT's) of less than about 40 seconds for normal platelet-rich plasma (the procedure for the activated plasma clotting time is discussed below). This consistency and time is achieved by including, along with the ellagic acid and phenol, an effective amount
10 of suitable metal ion. The suitable metal ions are preferably, but not necessarily, divalent, with preferred divalent metal ions being transition metal ions such as Cu^{2+} , Ni^{2+} , and Co^{2+} , and most preferably Ni^{2+} .

These suitable divalent metal ions are preferably provided by
15 their water soluble halogen or sulfate salts, such as cupric sulfate, nickel chloride, or cobalt chloride. Additionally, a preferred monovalent metal ion is Cu^{+} , and a preferred trivalent metal ion is Fe^{3+} . Ca^{2+} metal ions are less preferred, because calcium's presence in significant amount
20 in the reagent, and thus during incubation of the sample, tends to prematurely initiate clotting mechanisms, thus interfering with obtaining accurate clotting times.

As to the amounts of the ellagic acid and the suitable metal ion, any amounts by which substantial activation of the
25 intrinsic coagulation system can be achieved are acceptable. Preferably, the ellagic acid is included in molar concentrations of about 10^{-4} to 10^{-9} , and more preferably about 10^{-4} to 10^{-5} M. Additionally, it is preferred that the suitable metal ion be included in about an equal molar
30 concentration to the ellagic acid, although concentrations greater than equal molar concentrations are also acceptable. Further, the more preferred activators of this embodiment include the phenol in an amount of about 0.02 M.

A suitable buffer to maintain physiological pH is also
35 preferably included. This buffer is preferably TRIS,

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although other suitable buffers, such as HEPES, can be used.

The excellent sensitivity and activating ability of these inventive ellagic acid-based activators is born out by the excellent results obtained for clotting tests reported
5 further herein using these ellagic acid-based activators alone as the reagent, as well as by the superior aPTT reagents which are formed using these activators. Specific Examples 1-3 below further detail the preparation of intrinsic coagulation activators according to this
10 embodiment.

A second preferred embodiment is a method for preparing a coagulation test reagent. The method comprises the steps of (a) preparing an aqueous solution by dissolving ellagic acid in water in the presence of a suitable base, (b) adding
15 phenol to said aqueous solution, and (c) after said adding phenol, adding a suitable metal ion to said solution, said phenol being added in amounts sufficient to inhibit formation of insoluble metal hydroxides of said metal ion, whereby the overall reagent is a solution. Many suitable bases which aid
20 in the aqueous dissolution of ellagic acid are known, and accordingly could be used within the scope of this invention. However, the applicant's preferred base for this purpose is tetramethylammonium hydroxide. It is important that the ingredients be added in the order provided by this
25 method, as any other order leads to inferior reagents containing insoluble materials which interfere with uniformity of activation and consistency of result. Additional aspects of this embodiment, including those relating to the preferred amounts of ellagic acid and amounts
30 and types of metal ions, are analogous to those detailed in the first embodiment discussed above and can also be found in Examples 1-3 below.

Propyl Gallate and Tannin Coagulation Activators

As stated above, a third preferred embodiment relates to
35 the applicant's discovery that tannin and propyl gallate, in

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combination with a suitable metal ion, each provide a uniform, consistent activator of intrinsic coagulation. Accordingly, this third embodiment relates to a reagent for activating intrinsic coagulation, which reagent contains a
5 suitable metal ion and propyl gallate or tannin.

Although reagents in accordance with this embodiment need not be solutions to be effective, it is preferred that they be solutions in order to achieve the same significant advantages discussed in connection with the embodiments set
10 forth above. Propyl gallate and tannin provide advantage over ellagic acid and similar suitable hydroxy-substituted aromatic compounds, because they more readily dissolve and remain so dissolved in aqueous solutions.

The reagent of this embodiment also contains a suitable
15 metal ion. The metal ions need not be, but preferably are, divalent metal ions, with preferred suitable divalent metal ions being transition metal ions such as Ni^{2+} , Cu^{2+} and Co^{2+} , most preferably Ni^{2+} . As in the first embodiment, a preferred monovalent metal ion is Cu^+ , and a preferred
20 trivalent ion is Fe^{3+} . The tannin and propyl gallate, and the metal ion, can be included in the reagent in any amounts by which the reagent activates coagulation. Preferably, however, propyl gallate is included in the reagent in molar concentrations of about 10^{-9} to 10^{-2} , and more preferably
25 10^{-2} to 10^{-3} M, and tannin is preferably included in the reagent in amounts of about 10^{-5} to about 10^{-1} weight %, and more preferably about 0.005 weight %. The metal ions are preferably included in amounts of about 10^{-9} to 10^{-4} M, and more preferably about 10^{-4} to 10^{-5} M. Additionally,
30 the preferred divalent metal ions are preferably provided by their water soluble halogen or sulfate salts, such as cupric sulfate, nickel chloride, or cobalt chloride.

In addition to the above ingredients, the reagent of this embodiment also preferably includes a suitable buffer to
35 maintain physiological pH. Many acceptable buffers of this

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type are known, including for instance
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (also
known as HEPES) and Tris(hydroxymethyl)aminomethane (also
known as TRIS). The applicant's preferred buffer, however,
5 is TRIS, which is most preferably included in a concentration
of about 0.01 M.

Also, in one preferred mode, the reagent of this
embodiment includes platelet substitute, and accordingly is
an activated partial thromboplastin time reagent. The
10 preferred platelet substitute is ASOLECTIN, which is a soy
bean-derived phospholipid available from Associate
Concentrates, Inc. of Woodside N.Y., although many other
suitable platelet substitutes are available, including for
instance cephalin derived from rabbit or other animal brain.
15 Additional details regarding the preparation of reagents of
this embodiment are provided in Examples 7-9, 13-15, 19-21
and 28-30 below.

Highly Sensitive aPTT Reagents and Kits

As discussed above, prior aPTT reagents have been
20 criticized for their lack of sensitivity. The applicant has
developed aPTT reagents having far superior sensitivity than
prior reagents, and accordingly a fourth preferred embodiment
provides a reagent for activation of intrinsic coagulation
which comprises freeze-dried platelet substitute
25 reconstituted with an aqueous solution containing effective
activating amounts of (i) a suitable hydroxy-substituted
aromatic compound, and (ii) a suitable metal ion. This
overall inventive reagent is a solution, and gives consistent
aPTT values of less than about 40 seconds for normal platelet
30 poor plasma. Moreover, this reagent is highly sensitive,
which is demonstrated by the reagent giving aPTT values of at
least about 200 seconds for 1 u/ml heparinized normal
platelet poor plasma. It is preferred that the aqueous
solution be an activator as described in the first and third

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embodiments above. Further, the solution form of the overall reagent can be demonstrated by the similar aPTT values obtained before and after filtration, as is illustrated in Example 35 below.

- 5 To exemplify reagents according to this embodiment, aPTT tests were performed on normal human plasma using aPTT reagent which was prepared by reconstituting 0.001 g freeze-dried platelet substitute (ASOLECTIN) with 5 ml of the intrinsic coagulation activators ("Intrinsic Activators")
- 10 from the Examples indicated in Table 1 below. The aPTT tests were run in the usual manner by adding 0.1 ml of the prepared aPTT reagent to a 0.1 ml sample of citrated platelet-poor normal plasma. The plasma was then incubated at 37°C for 5 minutes, whereafter calcium chloride was added to the sample
- 15 initiate clotting, and the time necessary for the samples to clot was measured. The results of these tests are set forth in Table 1.

TABLE 1

| | <u>Intrinsic Activator</u> | <u>aPTT (sec.)</u> |
|----|-------------------------------|--------------------|
| 20 | Copper/ellagic acid (Ex. 1) | 24.4 |
| | Nickel/ellagic acid (Ex. 2) | 27.5 |
| | Cobalt/ellagic acid (Ex. 3) | 20.3 |
| | Copper/propyl gallate (Ex. 7) | 29.0 |
| | Nickel/propyl gallate (Ex. 8) | 29.4 |
| 25 | Cobalt/propyl gallate (Ex. 9) | 30.3 |
| | Copper/tannin (Ex. 13) | 30.7 |
| | Nickel/tannin (Ex. 14) | 29.1 |
| | Cobalt/tannin (Ex. 15) | 28.0 |

- In another set of experiments, the sensitivity of the
- 30 inventive aPTT reagents was compared to that of known commercial reagents. For instance, heparin sensitivity of a preferred aPTT reagent according to the invention and various commercial aPTT reagents was compared. Accordingly, 0.001 g

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of lyophilized ASOLECTIN platelet substitute ("PS") was reconstituted with 5 ml of the applicant's preferred Intrinsic Activator prepared in Example 2 (this aPTT reagent is indicated by "PS/5ml" in Table 2). Also, 0.001 g of lyophilized ASOLECTIN was reconstituted with only 2 ml of this Intrinsic Activator (indicated as "PS/2ml" in Table 2). These inventive aPTT reagents, and the commercial aPTT reagents identified in Table 2 below, were used to perform aPTT tests on normal human plasma and on heparinized (1 u/ml) normal human plasma. The results demonstrate that the inventive aPTT reagents have highly superior sensitivity to heparin, and gave aPTT values in excess of 200 seconds for 1 u/ml heparinized plasma, even ranging above 250 seconds. This superior sensitivity to heparin represents a significant increase over known aPTT reagents.

TABLE 2

| <u>aPTT Reagent</u> | <u>Normal Control</u> | <u>Heparinized</u> |
|-------------------------------|-----------------------|--------------------|
| PS/5ml | 30 sec. | 345 sec. |
| PS/2ml | 28 sec. | 245 sec. |
| Dade ACTIN | 28 sec. | 125 sec. |
| Ortho ACTIVATED THROMBOFAX | 22 sec. | 100 sec. |

In general, a reagent which has superior sensitivity to heparin will also have superior sensitivity to Factor deficiencies. In another set of experiments, this proved to be true for the inventive reagents. Plasma samples having Factor VIII, IX, and X deficiencies were assayed using an inventive aPTT reagent and commercial aPTT reagents. The inventive aPTT reagent was prepared by reconstituting 0.001 g of lyophilized ASOLECTIN platelet substitute "PS" with 10 ml of the Intrinsic Activator of Example 2 (this aPTT reagent is

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indicated as "PS/10 ml" in Table 3). The results of the respective aPTT tests are given in Table 3 below. As is shown, the inventive aPTT reagents gave aPTT values in excess of 150 seconds for the Factor deficient plasmas, even ranging above 200 seconds. These values represent a marked improvement over known aPTT reagents.

TABLE 3

| <u>10 Reagent</u> | <u>aPTT for Factor VIII deficiency</u> | <u>aPTT for Factor IX deficiency</u> | <u>aPTT for Factor X deficiency</u> |
|-------------------------------|--|--|---|
| PS/10ml | 212.5 | 249.0 | 203.2 |
| Dade ACTIN | 50.1 | 94.9 | 94.4 |
| Ortho ACTIVATED THROMBOFAX | 48.4 | 53.2 | 40.7 |

15 A fifth preferred embodiment relates to a coagulation test reagent kit from which the preferred coagulation activators of the fourth embodiment above can be conveniently and effectively prepared. This kit comprises (i) a vial containing freeze-dried platelet substitute, and (ii) an aqueous solution comprising effective amounts of (i) a suitable hydroxy-substituted aromatic compound and (ii) a suitable metal ion. The hydroxy-substituted aromatic compound and the metal ion are fully solubilized. Further, the aqueous solution and platelet substitute, when combined in controlled amounts, form a reagent which is also a solution and which gives consistent aPTT values of less than about 40 seconds for normal plasma, and which gives aPTT values of at least about 200 seconds for 1 u/ml heparinized normal platelet poor plasma. Preferably, the platelet substitute is included in the vial in an amount whereby reconstitution with about 2 ml to about 10 ml of the solution forms a reagent giving the indicated aPTT values. Further preferred aspects of this embodiment correspond to those of the fourth embodiment discussed above.

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The applicant has also discovered that the preferred aPTT reagents and kits according to the fourth and fifth embodiments above can be used in sensitive assays to detect lupus anticoagulant. Thus, these reagents and kits form the basis for a method for detecting the presence of lupus anticoagulant in subject plasma, which comprises the steps of (a) incubating platelet poor subject plasma in contact with a reagent according to the fourth embodiment above, (b) adding calcium to the platelet poor subject plasma in effective amounts to initiate clotting, and (c) measuring the time necessary for the platelet poor subject plasma to clot. Typically, aPTT times at least about 5 seconds greater than those obtained for normal controls can signify the presence of lupus anticoagulant. In the applicant's work, using this inventive method, lupus anticoagulant plasmas have typically exhibited aPTT times in excess of 50 seconds, and often in excess of 100 seconds or more.

Heparin Sensitive Reagents With Dextran Sulfate

As stated above, a sixth preferred embodiment relates to an improvement in a reagent for activation of intrinsic coagulation. In accordance with this embodiment, a coagulation activating reagent containing a suitable metal ion and a suitable hydroxy-substituted aromatic compound, also contains dextran sulfate in effective amounts to increase sensitivity of the reagent to heparin.

Although not required, it is preferred that the reagents according to this embodiment be solutions, as this term is defined above. Further, although other suitable hydroxy-substituted compounds can be used, reagents according to this embodiment preferably contain ellagic acid, propyl gallate or tannin. The respective preferred amounts of these three compounds in the reagents of this embodiment correspond to their preferred amounts in the reagents of embodiments described above. Similarly, the suitable metal ions are

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preferably divalent, with preferred suitable divalent metal ions being transition metal ions such as Ni^{2+} , Cu^{2+} and Co^{2+} , preferably included in the same amounts as in the embodiments discussed above.

5 Additionally, the reagents of this embodiment, in one preferred mode, also contain platelet substitute, and thus form heparin sensitive aPTT reagents. Again, the preferred platelet substitute is ASOLECTIN, and it is preferred that the overall reagent including the platelet substitute be a
10 solution.

 The dextran sulfate is preferably present in an amount between about 0.008 and 0.012 weight mg %, more preferably about 0.01 weight mg %, although any amount which increases sensitivity to heparin is sufficient. It is believed that
15 dextran sulfate neutralizes available PF4, thus leading to increased heparin sensitivity. Thus, other substances, such as protamine sulfate, which are equivalents to dextran sulfate and have substantially the same properties, are contemplated as being within the scope of the invention.

20 To verify significant increase in heparin sensitivity, heparinized plasma samples (1 u/ml) were tested for aPTT and aPCT using reagents with ("w/") and without ("w/o") dextran sulfate ("DS"). The results are set forth in Table 4 below. The composition and preparation of the preferred reagents
25 used is given in the specific Examples which are indicated in parentheses in Table 4. The "aPTT w/o DS" reagent indicated in Table 4 was prepared indentially to the reagent of Example 26, except without the addition of the dextran sulfate. The results demonstrate that the addition of
30 dextran sulfate substantially increases sensitivity to heparin.

TABLE 4

| | aPCT w/ DS (Ex. 5) | aPCT w/o DS (Ex. 2) | aPTT w/ DS (Ex. 26) | aPTT w/o DS |
|-------------------|-----------------------|------------------------|------------------------|-------------|
| <u>Plasma</u> | | | | |
| 35 Heparinized | 194.8 | 137.9 | 129.6 | 87.5 |
| Normal Control | 29.4 | 29.0 | 27.7 | 27.7 |

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Platelet-Sensitive Assays

A seventh preferred embodiment relates to a platelet-sensitive method for assaying plasma. This method includes the steps of (a) incubating the plasma in contact
5 with a reagent containing effective amounts of a suitable hydroxy-substituted aromatic compound and a suitable metal ion to cause the platelets to release Platelet Factor 3, (b) after the incubating, adding calcium to the plasma to initiate clotting, and (c) after the adding calcium,
10 measuring the time necessary for the plasma to clot, the plasma being essentially free from platelet substitute during clotting.

As the applicant has discovered, suitable hydroxy-substituted aromatic compounds and metal ions, which
15 were heretofore known to activate Hagemann factor, also cause platelets to release Platelet Factor 3. Accordingly, the method of this embodiment provides assays which are highly sensitive to platelets.

It is preferred that the reagent used in the incubating
20 step be a solution as defined above. Further, the hydroxy-substituted aromatic compound included in the reagent can be any suitable one as known in the art. Preferred, however, are ellagic acid, propyl gallate and tannin, or suitable derivatives thereof, with ellagic acid being most
25 preferred. These compounds are preferably included in the reagent in amounts corresponding to those disclosed above for other embodiments. Likewise, the metal ion, which is preferably a divalent transition metal ion (preferred again are Ni^{2+} , Cu^{2+} and Co^{2+} , most preferably Ni^{2+}), is
30 included in the same concentrations as discussed above for other embodiments. Of course, in order for the assay to be sensitive to platelets, the plasma being assayed must be at least essentially free from, and preferably free from platelet substitute during clotting. Accordingly, the
35 preferred reagents used in this embodiment are at least

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essentially free from, and more preferably free from platelet substitute. As indicated above, in several preferred modes, this platelet-sensitive method provides assays for platelet activity, systemic lupus, PF4, platelet reserve, which can
 5 signify excessive circulating low-density lipoproteins, and for platelet integrity. Each of these is discussed below and each provides an important assay to meet needs in this field which prior tests have not.

I. Activated Plasma Clotting Time (aPCT) Test

10 In this test, the platelet-sensitive method discussed above is performed on platelet-rich plasma. Accordingly, this test provides a sensitive and accurate measure of platelet activity of plasma. This test has been termed the "activated plasma clotting time" (aPCT) test by applicant.

15 APCT tests were performed on normal platelet-rich human plasma using preferred Intrinsic Activators of the invention as the aPCT reagents. Accordingly, a 0.1 ml amount of the Intrinsic Activator of the Example indicated in Table 5 below was added to a 0.1 ml sample of normal citrated platelet-rich
 20 plasma. The plasma was then incubated for 5 minutes, whereafter calcium chloride was added to the plasma to initiate clotting. The time necessary for the samples to clot was measured, and the results are given in Table 5 below.

25 TABLE 5

| <u>Intrinsic Activator</u> | <u>aPCT (sec.)</u> |
|----------------------------------|--------------------|
| Copper/ellagic acid (Ex. 1) | 30.3 |
| Nickel/ellagic acid (Ex. 2) | 30.0 |
| Cobalt/ellagic acid (Ex. 3) | 26.5 |
| 30 Copper/propyl gallate (Ex. 7) | 30.4 |
| Nickel/propyl gallate (Ex. 8) | 31.0 |
| Cobalt/propyl gallate (Ex. 9) | 29.4 |
| Copper/tannin (Ex. 13) | 32.4 |
| Nickel/tannin (Ex. 14) | 30.9 |
| 35 Cobalt/tannin (Ex. 15) | 32.9 |

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II. Determination of Platelet Reserve

In a normal subject, there is an excess of platelets beyond the quantity and quality required to support normal coagulation, which is herein referred to as the "platelet reserve". The applicant has discovered that the aPCT test can be used to accurately and reliably assay for platelet reserve. Accordingly, another preferred mode of this embodiment is an assay for determining platelet reserve in subject plasma. In this assay, the activated plasma clotting times of serial dilutions of platelet rich subject plasma with platelet poor subject plasma are determined. By analysis of the aPCT values obtained for the dilutions, a value representative of the platelet reserve can be determined.

For example, the platelet reserve can be determined by preparing dilutions of a patient's platelet rich plasma (PRP) with the patient's platelet poor plasma (PPP), and performing aPCT tests on the dilutions. Typically, the following dilutions can be used (PRP/PPP): 100% PRP; 50% PRP/50% PPP; 25% PRP/75% PPP; and 12.5% PRP/87.5% PPP. The aPCT times are then plotted on a graph having % PRP on one coordinate and aPCT values on the other. An aPTT test is performed on PPP, and the time obtained used as a baseline on the graph. Additionally, the aPCT value obtained using 100% platelet-rich plasma can be used as a baseline, although using this value as a baseline may in some instances lead to innaccurate results, such as where the patient has abnormally functioning platelets.

The applicant has found that the dilution curve will most often have linearity extending from 12.5% to 50% PRP, and almost always from 25% to 12.5% PRP. Thus, preferably a line is drawn through the aPCT values for the 25% and 12.5% PRP dilutions. This line is extended until it intersects the baseline. The % PRP corresponding to the point of

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intersection with the baseline is then subtracted from 100% to obtain the patient's platelet reserve.

FIG.'s 1-3 demonstrate platelet reserve determinations performed in accordance with this preferred embodiment. FIG. 1 shows a normal patient having a platelet reserve of 41% prior to aspirin therapy (line "A"). After four days of aspirin therapy at 1600 milligrams/day, the patient's platelet reserve was again determined, resulting in line "B". As can be seen, the patient's platelet reserve after the aspirin therapy decreased to 28%. FIG. 1 thus demonstrates that the platelet reserve is an excellent and sensitive test for monitoring aspirin therapy or other therapies which effect the activity of platelets. The above platelet reserve figures were calculated using an aPTT baseline. Using the aPCT value for 100% PRP as a baseline, a value of about 44% for platelet reserve prior to aspirin therapy is obtained, which is a good estimate of platelet reserve and does not require the additional step of performing an aPTT test.

FIG. 2 shows a platelet reserve determination on a patient on coumadin therapy. The patient's platelet reserve was 8%, well below the normal of about 30% to 40%. Thus, it was demonstrated that the platelet reserve test can also be performed on patients on coumadin or like therapy.

FIG. 3 shows a platelet reserve determination on a patient with defective Platelet Factor 3 release. As is shown, the patient's platelet reserve was determined to be 0%. Accordingly, the platelet reserve can also be used to detect functional defects of platelets.

In another experiment, it was demonstrated that a platelet reserve determination could be used to assay the functional integrity of platelets in a platelet concentrate. Accordingly, a platelet concentrate was obtained from Universal Reagents of Indianapolis, Indiana. The concentrate had a platelet count of 2,080,000/ml. This concentrate was

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diluted 50% with platelet poor plasma. This 50% diluted concentrate was then subjected to a platelet reserve assay as was performed for Fig.'s 1-3. The results are shown in FIG. 4, and demonstrate that the 50% dilution had a platelet reserve of 39%. Accordingly, taking into account the 50% dilution factor, the platelet concentrate was determined to have a platelet reserve of 78% ($39\% \times 2$). It will be understood that alternative analyses can be used to determine platelet reserve from the respective aPCT values obtained for the dilutions, and that graphical representation is unnecessary.

III. Detection of Lupus Anticoagulant

It has been discovered that the platelet-sensitive method provides a sensitive and specific assay for lupus anticoagulant when performed on platelet-poor plasma. For example, Table 6 summarizes studies which demonstrate this specificity and sensitivity. Table 6 shows the clotting times obtained for various abnormal plasmas, including lupus plasmas (platelet poor), using the intrinsic activator of Example 2 (Ni/ellagic acid) as the reagent, as compared to aPTT values obtained for these same plasmas (using an aPTT reagent prepared as in Example 26 except without the addition of dextran sulfate). The variety of plasma's were obtained from Universal Reagents of Indianapolis, Indiana. As is demonstrated, the lupus test of this preferred mode has outstanding specificity and sensitivity for and to lupus anticoagulant.

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TABLE 6

| | <u>Type of Plasma</u> | <u>aPTT</u> | <u>aPCT</u> |
|----|-----------------------|-------------|-------------|
| | platelet rich plasma | 25.8 | 26.0 |
| | platelet poor plasma | 26.2 | 71.2 |
| 5 | Lupus anticoagulant | 39.0 | 263.1 |
| | Lupus anticoagulant | 48.7 | 269.5 |
| | ANA speckled | 39.7 | 67.4 |
| | ANA speckled | 32.2 | 62.9 |
| | ANA homogenous | 33.8 | 65.3 |
| 10 | ANA homogenous | 32.2 | 54.6 |
| | nDNA Antibody | 33.9 | 75.7 |
| | SCL-70 | 34.3 | 46.8 |
| | RNP | 44.7 | 71.2 |
| | SSA Antibody | 19.9 | 37.0 |
| 15 | SSB Antibody | 37.7 | 61.5 |

Table 7 below compares the lupus anticoagulant sensitivity of the applicant's preferred lupus test (using the Intrinsic Activator of Example 2 as the reagent), to the sensitivity obtained using various commercial aPTT reagents.

20 The plasmas tested were all platelet poor. As shown, the preferred lupus test of this mode is far superior than commercial aPTT reagents and tests in the detection of lupus anticoagulant.

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TABLE 7

| | <u>Type of Plasma</u> | <u>Ex. 2</u> | <u>Ortho aPTT</u> | <u>Dade ACTIN</u> | <u>Biodata</u> |
|---|---------------------------------------|--------------|-----------------------|-----------------------|----------------|
| | Lupus anticoagulant | 174.4 | 31.6 | 44.7 | 51.1 |
| 5 | Lupus anticoagulant | 221.9 | 32.6 | 46.1 | 61.0 |
| | Norm. Human Plasma (platelet poor) | 64.8 | 25.3 | 27.9 | 32.8 |

IV. Assay For Lipoprotein Defect

It has been demonstrated that the applicant's preferred
 10 platelet-sensitive method can be used to signify possible
 Type II lipoprotein defects. Subjects having this condition
 have excessively high circulating low-density lipoproteins.
 In this assay, the platelet-sensitive method is performed on
 platelet-poor subject plasma and on platelet-rich subject
 15 plasma (this latter test is simply an aPCT test). The two
 measured clotting times are then compared, whereby the lack
 of a significant difference can signify excessive circulating
 low-density lipoprotein.

For example, such an assay was performed on a patient
 20 suspected of having a Type II lipoprotein defect. The
 patient's platelet rich plasma exhibited an aPCT in
 accordance with the invention of 31.2 seconds (using the
 intrinsic activator of Example 2 as the aPCT reagent). The
 patient's platelet poor plasma demonstrated a clotting time
 25 of 33.1 seconds using the same reagent, as opposed to normal
 values of about 60 seconds or more for normal platelet poor
 plasma. A possible Type II lipoprotein defect was thus
 signified, which was confirmed by lipoprotein electrophoresis
 of the patient's plasma.

30 V. Assay For Platelet Factor 4

The platelet-sensitive method can also be used to test
 for Platelet Factor 4 release in plasma. In the PF4 assay,

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the platelet-sensitive method is performed on plasmas having varying ratios of platelets to heparin units, whereby neutralization of the heparin present by Platelet Factor 4 released by the platelets can be observed. Of course, the
5 varying ratios can be obtained by adjusting the level of platelets, or the level of heparin, or by adjusting the level of both.

For example, in one method, platelet rich subject plasma and at least one dilution of platelet rich subject plasma
10 (with platelet poor plasma) are equally heparinized. The measured clotting times are then compared, whereby a significantly lower clotting time for the heparinized platelet rich plasma as compared to the dilution can indicate release of sufficient Platelet Factor 4 in the platelet rich
15 plasma to neutralize the heparin. In an experiment using this approach, two plasma samples, each containing 1 unit of heparin per ml, were prepared. The first sample was 100% platelet rich plasma, and the second was 50% platelet rich plasma and 50% platelet poor plasma. The clotting times
20 obtained using the intrinsic activator of Example 2 as a reagent were 31.3 seconds for the 100% PRP, and 208.6 seconds for the 50% dilution. The relatively short clotting time for the 100% PRP indicates the release of sufficient PF4 to neutralize the heparin. The relatively long clotting time
25 for the 50% dilution indicates that less PF4 has been released (due to the reduced concentration of platelets) so that less heparin was neutralized, leading to the longer clotting time. The ability of the plasma used in the two samples to release PF4 was thus demonstrated. Had the plasma
30 had a defect in PF4 release or function, the aPCT value for the 100% PRP would have been significantly longer.

It will be appreciated that significantly more precise measures of the levels of PF4 in plasma samples can be obtained by assaying plasmas having several ratios of
35 platelets to heparin to determine more precisely the point of

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neutralization of the heparin by PF4. Accordingly, in another experiment, dilutions were prepared having effectively the levels of heparin per ml of PRP indicated in Table 8 below. The clotting times were obtained for each of these dilutions using the intrinsic activator of Example 2 as the reagent. The results are tabulated in Table 8, and graphically represented in Fig. 5. These results demonstrate the sufficient release of PF4 in the patient's platelet-rich plasma to neutralize between 3 and 4 units of heparin per ml. Thus the inventive PF4 assay provides a way to accurately determine PF4 levels in plasma samples.

TABLE 8

| | <u>U/Heparin/ml</u> | <u>Clotting Time (sec)</u> |
|----|---------------------|----------------------------|
| | 0 | 30.1 |
| 15 | 0.5 | 30.1 |
| | 1.0 | 29.6 |
| | 1.5 | 28.8 |
| | 2.0 | 27.3 |
| | 3.0 | 39.4 |
| 20 | 4.0 | 186.8 |

Activated Whole Blood Clotting Time

The applicant has also demonstrated that the preferred intrinsic activators can be used in performing activated whole blood clotting time tests (A.W.B.C.T). Accordingly, the activated whole blood clotting time of fresh human blood was determined by incubating 0.1 ml fresh human blood and 0.1 ml of the intrinsic activator of Example 2 in a Fibrometer for 5 minutes. 0.1 ml 0.02 M CaCl_2 was added, whereafter the A.W.B.C.T. value obtained was 43.4 seconds. Similar tests on two additional fresh whole blood samples gave A.W.B.C.T. values of 38.4 and 38.9 seconds. Thus the inventive Intrinsic Activators are useful for whole blood as well as plasma-based tests.

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Method and Materials to Stem Bleeding

In yet another set of experiments, the applicant has discovered that bleeding from wounds can be stopped up to six time faster by applying to the bleeding site an effective amount of a hemostatic agent including a suitable metal ion and a hydroxy-substituted aromatic compound. Preferred are reagents according to the first embodiment above, which can in this embodiment also optionally include a platelet substitute, preferably ASOLECTIN, and also preferred are reagents according to the third embodiment above.

For example, in one experiment the intrinsic activator of Example 2 was applied to a portion of fibrous material (e.g. filter paper) and this material was then applied to a bleeding finger wound. The wound stopped bleeding in about 16 seconds. Another bleeding finger wound treated similarly with an unmodified portion of the fibrous material stopped bleeding only after 94 seconds. It was thus demonstrated that the method of this embodiment provides an outstanding way to stem bleeding.

It will be understood that there are other materials to which the reagents can be applied in particular bleeding applications. For instance, it is known to prepare pastes by reconstituting cryoprecipitates of human blood with thromboplastin for application to bleeding sites during surgery such as coronary surgery. In one preferred mode of this embodiment, the indicated reagents are used in the reconstitution of the cryoprecipitates in the preparation of such pastes. The resulting pastes have superior ability to slow or stop bleeding, and thus these materials themselves constitute a further inventive embodiment.

The following Examples are given in order to further illustrate the invention.

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EXAMPLE 1**ELLAGIC ACID AND COPPER II INTRINSIC ACTIVATOR**

0.18 g tetramethylammonium hydroxide were dissolved in 1,000 ml water. 0.034 g ellagic acid were then dissolved in
5 this tetramethylammonium hydroxide solution. 1.5 g phenol were then dissolved in the tetramethylammonium hydroxide-ellagic acid solution, whereafter 1.0 ml 0.1 M cupric sulfate were added and the resulting solution mixed for 10 minutes with teflon coated stirring bar on a
10 stir-plate. 1.2 g TRIS buffer were then dissolved into the solution. The resulting solution was clear and free from any visible particulate or other suspended matter.

EXAMPLE 2**ELLAGIC ACID AND NICKEL INTRINSIC ACTIVATOR**

15 Example 1 was repeated except 1.0 ml 0.1 M nickel chloride was used instead of the 1.0 ml 0.1 M cupric sulfate to form an ellagic acid/nickel coagulation activator which was also a clear solution free from visible particulate or other suspended matter.

EXAMPLE 3**ELLAGIC ACID AND COBALT INTRINSIC ACTIVATOR**

20 Example 1 was repeated except 1.0 ml 0.1 M cobalt chloride was used instead of the 1.0 ml 0.1 M cupric sulfate, and the TRIS buffer was not added. A clear ellagic
25 acid/cobalt coagulation activator solution was formed. This unbuffered activator performs similarly to the buffered activators in Examples 1 and 2. The buffer further stabilizes the activators over time.

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EXAMPLE 4
HEPARIN SENSITIVE INTRINSIC ACTIVATOR
WITH ELLAGIC ACID, DEXTRAN SULFATE, AND COPPER II

Example 1 was repeated except 1.0 ml 100 mg/dl dextran sulfate was added along with the cupric sulfate to form a coagulation activator sensitive to heparin.

EXAMPLE 5
HEPARIN SENSITIVE INTRINSIC ACTIVATOR
WITH ELLAGIC ACID, DEXTRAN SULFATE, AND NICKEL

Example 2 was repeated except 1.0 ml 100 mg/dl dextran sulfate was added along with the nickel chloride. A heparin sensitive coagulation activator with ellagic acid, dextran sulfate, and nickel was thus formed.

EXAMPLE 6
HEPARIN SENSITIVE INTRINSIC ACTIVATOR
WITH ELLAGIC ACID, DEXTRAN SULFATE AND COBALT

Example 3 was repeated except 1.0 ml 100 mg/dl dextran sulfate was added along with the cobalt chloride to form a heparin sensitive coagulation activator with ellagic acid and cobalt.

EXAMPLE 7
PROPYL GALLATE AND COPPER II INTRINSIC ACTIVATOR

0.5 g propyl gallate were dissolved in 1,000 ml water. 1.0 ml 0.1 M cupric sulfate were then added, whereafter the solution was mixed for 10 minutes with teflon-coated stir bar. 1.2 g TRIS buffer were then dissolved in this solution, which was thereafter stirred for an additional 10 minutes. A propyl gallate/copper coagulation activator was formed as a clear solution without any visible suspended materials.

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EXAMPLE 8
PROPYL GALLATE AND NICKEL INTRINSIC ACTIVATOR

Example 7 was repeated except 1.0 ml 0.1 M nickel chloride was used instead of the cupric sulfate. A clear solution-form coagulation activator with propyl gallate and nickel was formed.

EXAMPLE 9
PROPYL GALLATE AND COBALT INTRINSIC ACTIVATOR

Example 7 was repeated except 1.0 ml 0.1 M cobalt chloride was used the place of the cupric sulfate to form a propyl gallate/cobalt solution-form coagulation activator.

EXAMPLE 10
HEPARIN SENSITIVE INTRINSIC ACTIVATOR
WITH PROPYL GALLATE, DEXTRAN SULFATE AND COPPER II

Example 7 was repeated except 1.0 ml 100 mg/dl dextran sulfate was added along with the cupric sulfate. A heparin sensitive coagulation activator with propyl gallate, dextran sulfate and copper was thus formed.

EXAMPLE 11
HEPARIN SENSITIVE INTRINSIC ACTIVATOR
WITH PROPYL GALLATE, DEXTRAN SULFATE AND NICKEL

Example 8 was repeated except 1.0 ml 100 mg/dl dextran sulfate was added along with the nickel chloride to form a heparin sensitive coagulation activator containing propyl gallate, dextran sulfate and nickel.

EXAMPLE 12
HEPARIN SENSITIVE COAGULATION ACTIVATOR
WITH PROPYL GALLATE, DEXTRAN SULFATE AND COBALT

The procedure of Example 9 was repeated except 1.0 ml 100 mg/dl dextran sulfate was added along with the cobalt chloride. A solution-form coagulation activator was thus formed which was sensitive to heparin and contained propyl gallate, dextran sulfate, and cobalt.

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EXAMPLE 13
TANNIN AND COPPER II INTRINSIC ACTIVATOR

0.5 g tannin were dissolved in 1,000 ml reagent water.
1.0 ml 0.1 M cupric sulfate were added whereafter the
5 resulting solution was mixed for 10 minutes with a teflon
coated stirring bar on a stir-plate. Then, 1.2 g TRIS buffer
were added, and the resulting solution mixed for 10 minutes
with a teflon coated stir-bar. A clear solution coagulation
activator with tannin and copper was thus formed containing
10 no visible suspended matter.

EXAMPLE 14
TANNIN AND NICKEL INTRINSIC ACTIVATOR

Example 13 was repeated except 1.0 ml 0.1 M Nickel
chloride was used in the place of the cupric sulfate thus
15 forming a solution-form coagulation activator with tannin and
nickel.

EXAMPLE 15
TANNIN AND COBALT INTRINSIC ACTIVATOR

Example 13 was repeated except 0.1 ml 0.1 M cobalt
20 chloride was used instead of the cupric sulfate. Also, 2,5 g
HEPES hemi sodium salt were added instead of the TRIS, to
thus make a coagulation activator in solution form containing
tannin and cobalt.

EXAMPLE 16
HEPARIN SENSITIVE INTRINSIC ACTIVATOR
WITH TANNIN, DEXTRAN SULFATE AND COPPER II

25 Example 13 was repeated, but 1.0 ml 100 mg/dl dextran
sulfate was added along with the cupric sulfate to form a
heparin sensitive coagulation activator solution containing
30 tannin, dextran sulfate and copper.

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EXAMPLE 17
HEPARIN SENSITIVE INTRINSIC ACTIVATOR
WITH TANNIN, DEXTRAN SULFATE AND NICKEL

Example 14 was repeated except 1.0 ml 100 mg/dl dextran sulfate was added along with the nickel chloride. A heparin sensitive coagulation activator solution containing tannin, dextran sulfate and nickel was formed.

EXAMPLE 18
HEPARIN SENSITIVE INTRINSIC ACTIVATOR
WITH TANNIN, DEXTRAN SULFATE AND COBALT

Example 15 was repeated, but 1.0 ml 100 mg/dl dextran sulfate was added along with the cobalt chloride to make a heparin sensitive coagulation activator with tannin, dextran sulfate and cobalt.

EXAMPLE 19
APTT REAGENT INCLUDING
PROPYL GALLATE AND COPPER II ACTIVATOR

In this example, a solution-form aPTT reagent was formed which included a propyl gallate and copper coagulation activator. Accordingly, 0.5 g propyl gallate were dissolved in 1,000 ml reagent water. 1.2 g TRIS buffer were then dissolved in this solution whereafter it was mixed for 10 minutes with a teflon coated stirring bar on a stir-plate. 1.0 g Asolectin and 200 ml above solution were placed in a Waring blender and blended at full speed for 1.0 minute. The resulting emulsion was returned to the original solution and mixed for 10 minutes, whereafter 1.0 ml 0.1 M cupric sulfate was added and the resulting solution was mixed for an additional 10 minutes. A solution-form aPTT reagent was formed which was clear and free from the cloudiness or suspended material commonly observed in commercial aPTT reagents.

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EXAMPLE 20
APTT REAGENT CONTAINING
PROPYL GALLATE AND NICKEL ACTIVATOR

Example 19 was repeated except 1.0 ml 0.1 M nickel
5 chloride was used instead of the cupric sulfate, thus forming
a solution-form aPTT reagent containing a propyl gallate and
nickel coagulation activator.

EXAMPLE 21
APTT REAGENT CONTAINING
10 **PROPYL GALLATE AND COBALT ACTIVATOR**

Example 19 was repeated except 1.0 ml 0.1 M cobalt
chloride was used in the place of the cupric sulfate. A
solution-form aPTT reagent containing a propyl gallate and
cobalt activator was thus formed.

EXAMPLE 22
15 **HEPARIN SENSITIVE APTT REAGENT CONTAINING**
DEXTRAN SULFATE AND A PROPYL GALLATE/COPPER II ACTIVATOR

The preparation of Example 19 was repeated except 1.0 ml
100 mg/dl dextran sulfate was added along with the cupric
20 sulfate to render the aPTT reagent heparin sensitive. The
reagent formed was a solution containing the propyl
gallate/copper activator.

EXAMPLE 23
25 **HEPARIN SENSITIVE APTT REAGENT CONTAINING**
DEXTRAN SULFATE AND A PROPYL GALLATE/NICKEL ACTIVATOR

Example 20 was repeated except 1.0 ml 100 mg/dl dextran
sulfate was added along with the nickel chloride.
Accordingly, a solution-form aPTT reagent was prepared which
was sensitive to heparin and which contained a propyl
30 gallate/nickel activator.

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EXAMPLE 24
HEPARIN SENSITIVE APTT REAGENT CONTAINING
DEXTRAN SULFATE AND A PROPYL GALLATE/COBALT ACTIVATOR

The preparation of Example 21 was repeated, but 1.0 ml
5 100 mg/dl dextran sulfate was added with the cobalt
chloride. A solution-form aPTT reagent was formed containing
a propyl gallate/cobalt coagulation activator and which was
sensitive to heparin.

EXAMPLE 25
HEPARIN SENSITIVE APTT REAGENT CONTAINING
10 **DEXTRAN SULFATE AND AN ELLAGIC ACID/COPPER II ACTIVATOR**

0.18 g tetramethylammonium hydroxide were dissolved in
1,000 ml reagent water. 0.034 g ellagic acid were dissolved
in the tetramethylammonium hydroxide solution, after which
15 1.5 g phenol were dissolved in the tetramethylammonium
hydroxide-ellagic acid solution. 1.0 g Asolectin and 200 ml
of above solution were then added to a Waring blender and
blended for 1 minute at top speed. The resulting emulsion
was then returned to the original solution and mixed for 10
20 minutes with a teflon coated stirring bar on a stir-plate,
whereafter 1.0 ml 0.1 M cupric sulfate and 1.0 ml 100 mg/dl
dextran sulfate were added and the resulting solution mixed
for 10 minutes more. The resulting heparin sensitive aPTT
reagent containing an ellagic acid/copper activator was a
25 solution without any visible suspended material. Another
identical reagent was prepared, except 1.2 g TRIS buffer
were added in the final step.

EXAMPLE 26
HEPARIN SENSITIVE APTT REAGENT CONTAINING
30 **DEXTRAN SULFATE AND AN ELLAGIC ACID/NICKEL ACTIVATOR**

Example 25 was repeated except 1.0 ml 0.1 M nickel
chloride was used in place of the cupric sulfate. A
solution-form heparin sensitive aPTT reagent with an ellagic
acid/nickel activator was thus prepared.

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EXAMPLE 27
HEPARIN SENSITIVE APTT REAGENT CONTAINING
DEXTRAN SULFATE AND AN ELLAGIC ACID/COBALT ACTIVATOR

Example 25 was repeated except 1.0 ml 0.1 M cobalt
5 chloride was used instead of the cupric sulfate to prepare a
solution-form heparin sensitive aPTT reagent with an ellagic
acid/cobalt activator.

EXAMPLE 28
APTT REAGENT CONTAINING
A TANNIN/COPPER II COAGULATION ACTIVATOR

10

0.5 g tannin were dissolved in 1,000 ml reagent water.
4.9 g HEPES 0.5 sodium were added to the resulting solution
which was then mixed for 10 minutes with a teflon coated
stirring bar on a stir-plate. 1.0 g Asolectin and 200 ml of
15 the above solution were then placed in a Waring blender and
blended at full speed for 1.0 minute. The resulting emulsion
was returned to the original solution and mixed for 10
minutes. Thereafter, 1.0 ml 0.1 M cupric sulfate was added
and the solution mixed for another 10 minutes. Formed was an
20 aPTT reagent in solution form free from visible suspended
materials, and containing a tannin/copper coagulation
activator.

EXAMPLE 29
APTT REAGENT CONTAINING
A TANNIN/NICKEL COAGULATION ACTIVATOR

25

Example 28 was repeated except 1.0 ml 0.1 M nickel
chloride was used instead of the cupric sulfate. A
solution-form aPTT reagent was thus prepared having a
tannin/nickel coagulation activator.

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EXAMPLE 30
APTT REAGENT CONTAINING
A TANNIN/COBALT COAGULATION ACTIVATOR

Example 28 was repeated except 1.0 ml 0.1 M cobalt
5 chloride was used instead of the cupric sulfate. A
solution-form aPTT reagent was thus prepared having a
tannin/cobalt coagulation activator.

EXAMPLE 31
HEPARIN SENSITIVE APTT REAGENT CONTAINING
10 **DEXTRAN SULFATE AND A TANNIN/COPPER II ACTIVATOR**

Example 28 was repeated except 1.0 ml 100 mg/dl dextran
sulfate was added along with the cupric sulfate. A heparin
sensitive solution-form aPTT reagent was thus formed having a
tannin/copper activator.

EXAMPLE 32
15 **HEPARIN SENSITIVE APTT REAGENT CONTAINING**
DEXTRAN SULFATE AND A TANNIN/NICKEL ACTIVATOR

Example 29 was repeated except 1.0 ml 100 mg/dl dextran
sulfate was added with the nickel chloride to form a heparin
20 sensitive aPTT reagent containing a tannin/nickel activator
and being in solution form.

EXAMPLE 33
HEPARIN SENSITIVE APTT REAGENT CONTAINING
DEXTRAN SULFATE AND A TANNIN/COBALT ACTIVATOR

25 Example 30 was repeated, but with 1.0 ml 100 mg/dl
dextran sulfate being added along with the cobalt chloride.
A solution-form heparin sensitive aPTT reagent was thus
formed including a tannin/cobalt coagulation activator.

EXAMPLE 34
30 **INTRINSIC ACTIVATORS ARE IN SOLUTION**

An amount of a coagulation activator of Example 2 was
filtered through a 0.45 micron MILLIPORE filter, whereupon no

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residue on the filter was observed. Using the filtered coagulation activator, an aPCT test was performed on normal platelet rich plasma. The aPCT value using the filtered coagulation activator was 29.3 seconds. A similar aPCT was
5 run using an unfiltered amount of the same coagulation activator, and a value of 29.4 seconds was obtained. It was thus demonstrated that the activator was in solution form.

EXAMPLE 35
SOLUTION FORM OF INVENTIVE aPTT REAGENTS

10 0.001 g of ASOLECTIN was reconstituted with 5 ml of the coagulation activator of Example 2. The resulting aPTT reagent was then filtered through a 0.45 micrometer MILLIPORE filter whereupon no residue was observed. An aPTT value of
15 filtered reagent. Using a similarly prepared but non-filtered aPTT reagent, an aPTT value of 27.5 was obtained for normal platelet rich plasma, thus demonstrating that the total aPTT reagent is soluble.

20 While the inventions have been described in detail in the foregoing description, the same is to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiments have been described, and that all changes and modifications that come within the spirit of the invention are desired to be
25 protected.

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WHAT IS CLAIMED IS:

1. A reagent for uniform activation of intrinsic coagulation, consisting essentially of an aqueous solution of effective amounts of ellagic acid, phenol, and suitable metal ion, whereby the ellagic acid and metal ion are fully solubilized and consistent activated plasma clotting times of less than about 40 seconds can be achieved for normal plasma controls.
2. The reagent of claim 1 wherein at least about 90% of the reagent's activity is retained after filtration through a 0.45 micron filter.
3. The reagent of claim 1 in which the metal ion is a divalent metal ion.
4. The reagent of claim 3 in which the metal ion is a transition metal ion.
5. The reagent of claim 4 in which the metal ion is selected from the group consisting of Ni^{2+} , Co^{2+} , and Cu^{2+} .
6. The reagent of claim 5 in which the metal ion is Ni^{2+} .
7. The reagent of claim 5 in which the metal ion is Co^{2+} .
8. The reagent of claim 5 in which the metal ion is Cu^{2+} .
9. The reagent of claim 5 in which the metal ion is included in an amount of about 10^{-9} to about 10^{-4} M.

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10. The reagent of claim 5 in which the ellagic acid is included in an amount of about 10^{-9} to about 10^{-4} M.

11. The reagent of claim 10 in which the ellagic acid and the metal ion are each included in an amount of about 10^{-4} to about 10^{-5} M.

12. The reagent of claim 11 in which the phenol is included in an amount of about 0.02 M.

13. The reagent of claim 12 wherein at least about 90% of the reagent's activity is retained after filtration through a 0.45 micron filter.

14. The reagent of claim 13 wherein a level approaching 100% of the reagent's activity is retained after filtration through a 0.45 micron filter.

15. A method for preparing a coagulation reagent, comprising the steps of (a) preparing an aqueous solution by dissolving ellagic acid in water in the presence of a suitable base, (b) adding phenol to said aqueous solution, and (c) after said adding phenol, adding a suitable metal ion to said solution, said phenol being added in amounts sufficient to inhibit formation of insoluble metal hydroxides of said metal ion, whereby the overall reagent is a solution.

16. The method of claim 15 in which the base is tetramethylammonium hydroxide and the suitable metal ion is selected from the group consisting of Ni^{2+} , Co^{2+} , and Cu^{2+} .

17. In a reagent for activation of intrinsic coagulation, which reagent contains a suitable metal ion and

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a hydroxy-substituted aromatic compound, the improvement which comprises including propyl gallate or tannin as the hydroxy-substituted aromatic compound.

18. A reagent for activation of intrinsic coagulation, comprising a freeze-dried platelet substitute reconstituted with an aqueous solution containing effective amounts of (i) a suitable hydroxy-substituted aromatic compound and (ii) suitable metal ion, the overall reagent being a solution and giving consistent aPTT values of less than about 40 seconds for normal plasma, and giving aPTT values of at least about 200 seconds for 1 u/ml heparinized normal platelet poor plasma.

19. The reagent of claim 18 in which the hydroxy-substituted aromatic compound is selected from the group consisting of ellagic acid, propyl gallate and tannin.

20. The reagent of claim 19 in which the metal ion is selected from the group consisting of Ni^{2+} , Co^{2+} and Cu^{2+} .

21. The reagent of claim 20 wherein the overall reagent is a solution.

22. The reagent of claim 21 in which the hydroxy-substituted aromatic compound is ellagic acid.

23. A coagulation test reagent kit, comprising:
a vial containing freeze-dried platelet substitute; and
an aqueous solution comprising effective amounts of (i) a suitable hydroxy-substituted aromatic compound and (ii) a dissolved suitable metal ion, whereby the hydroxy-substituted aromatic compound and the metal ion are fully solubilized;
the solution and the platelet substitute when combined in

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predetermined amounts forming a reagent which is also a solution and which gives consistent aPTT values of less than about 40 seconds for normal platelet poor plasma, and which gives aPTT values of at least about 200 seconds for 1 u/ml
5 heparinized normal platelet poor plasma.

24. The kit of claim 23 in which the platelet substitute is included in the vial in an amount whereby when combined with about 2 to about 10 ml of the solution, the overall reagent gives said aPTT values.

10 25. The reagent of claim 24 in which the hydroxy-substituted aromatic compound is selected from the group consisting of ellagic acid, propyl gallate and tannin.

26. The reagent of claim 25 in which the metal ion is selected from the group consisting of Ni^{2+} , Co^{2+} and
15 Cu^{2+} .

27. The reagent of claim 26 in which the hydroxy-substituted aromatic compound is ellagic acid.

28. A method for detecting the presence of lupus anticoagulant in subject plasma, comprising the steps of:
20 incubating platelet poor subject plasma in contact with a reagent according to claim 18;

after said incubating, adding calcium to the plasma in effective amounts to initiate clotting; and

after said adding, measuring the time necessary for the
25 platelet poor subject plasma to clot.

29. In a reagent for activation of intrinsic coagulation, which reagent includes a suitable metal ion and a suitable hydroxy-substituted aromatic compound, the improvement which comprises including dextran sulfate in the

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reagent in effective amounts to increase sensitivity to heparin.

30. The reagent of claim 29 in which the hydroxy-substituted aromatic compound is selected from the group consisting of ellagic acid, propyl gallate and tannin.

31. The reagent of claim 30 in which the metal ion is selected from the group consisting of Ni^{2+} , Co^{2+} and Cu^{2+} .

32. The reagent of claim 31 in which the hydroxy-substituted aromatic compound is ellagic acid.

33. A platelet-sensitive method for assaying coagulative properties of plasma, comprising the steps of:

incubating at least one sample of the plasma in contact with a reagent containing effective amounts of a suitable hydroxy-substituted aromatic compound and a suitable metal ion to cause the platelets to release Platelet Factor 3;

after the incubating, adding calcium to the sample to initiate clotting; and

after the adding calcium, measuring the time necessary for the sample to clot, the sample being at least essentially free from platelet substitute during clotting.

34. The method of claim 33 in which the hydroxy-substituted aromatic compound is selected from the group consisting of ellagic acid, propyl gallate and tannin.

35. The method of claim 34 in which the hydroxy-substituted aromatic compound is ellagic acid.

36. The method of claim 33 in which the sample is platelet rich plasma, whereby the method provides an activated plasma clotting time assay.

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37. The method of claim 33 in which said incubating, adding and measuring steps are applied to at least two samples, each sample representing a serial dilution of platelet rich plasma with platelet poor plasma, the method
5 further comprising the step of determining by analysis of the respective clotting times obtained for the samples, a value representative of the platelet reserve.

38. The method of claim 33 in which the sample is platelet poor plasma, whereby the method provides a sensitive
10 assay for lupus anticoagulant.

39. The method of claim 33 in which the incubating, adding and measuring steps are applied to at least two samples, one sample being platelet rich plasma and another being platelet poor plasma, the method further comprising the
15 step of comparing the clotting times obtained for the two samples, whereby the lack of significant difference between the clotting times can signify excessive circulating low-density lipoprotein.

40. The method of claim 33 in which the incubating, adding and measuring steps are applied to at least two
20 samples having differing ratios of platelets to heparin units, the method further comprising the step of comparing the clotting times obtained for the two samples, whereby a significantly lower clotting time for the sample with the
25 greater platelet to heparin unit ratio can indicate release of sufficient Platelet Factor 4 to neutralize the heparin.

41. A method for stemming bleeding from a bleeding site, comprising the step of applying to the bleeding site an effective amount of a hemostatic agent including a suitable
30 metal ion and a hydroxy-substituted aromatic compound.

= BEFORE TAKING ASPIRIN
= AFTER TAKING ASPIRIN FOR 4 DAYS (1600 MGL DAY)
PLATELET RESERVE BEFORE ASPIRIN = 41%
PLATELET RESERVE AFTER ASPIRIN = 28%

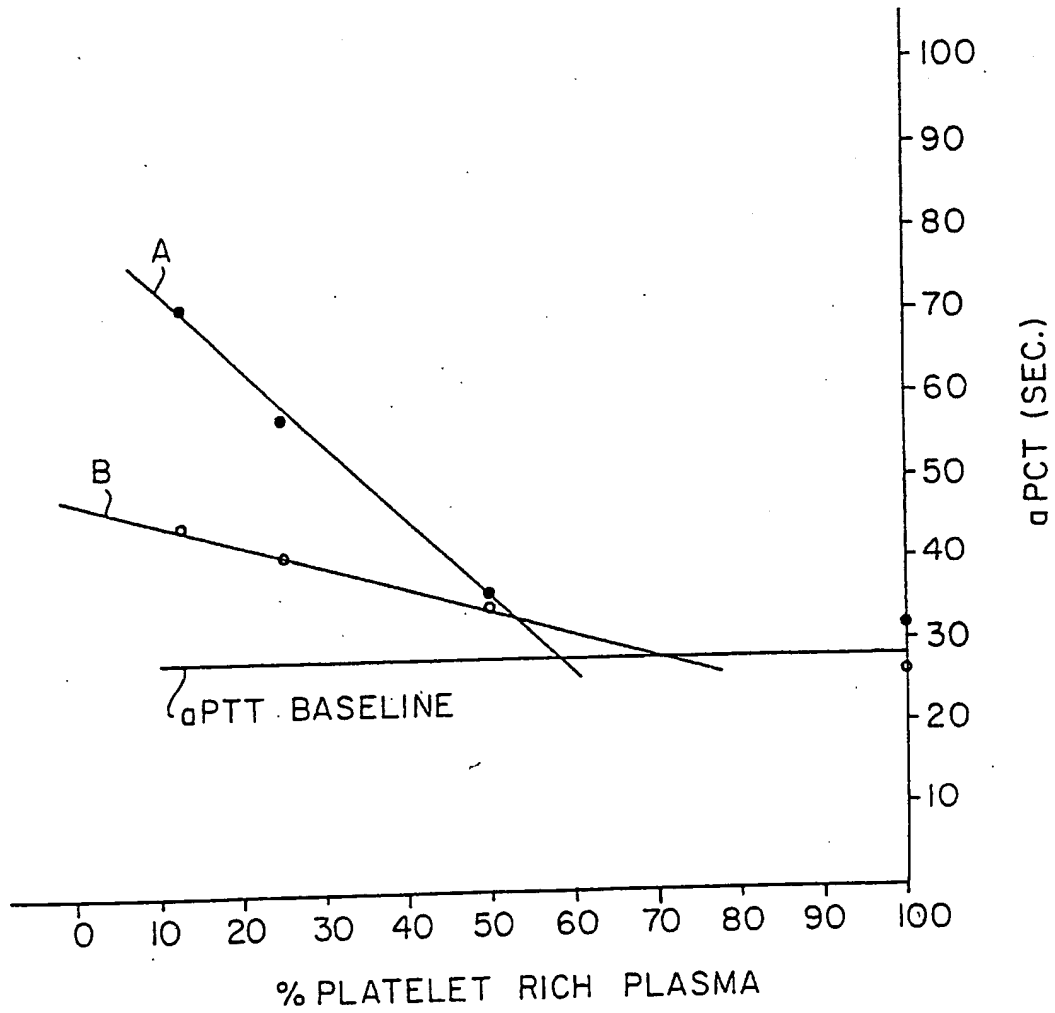


Fig.1

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PATIENT WITH ARTIFICIAL HEART VALVE ON COUMADIN
THERAPY

PLATELET RESERVE = 8 %

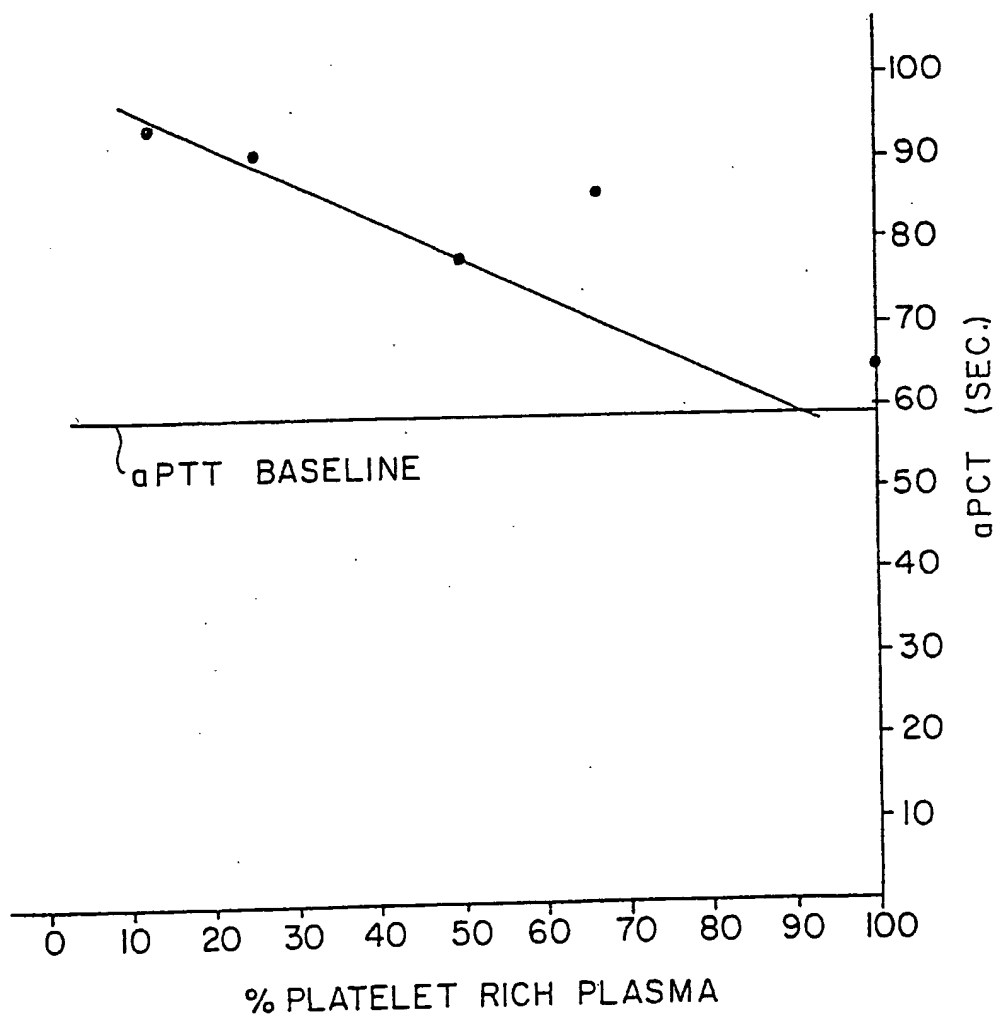


Fig.2

PATIENT WITH DEFECTIVE RELEASE OF PLATELET
FACTOR 3

PLATELET RESERVE = 0

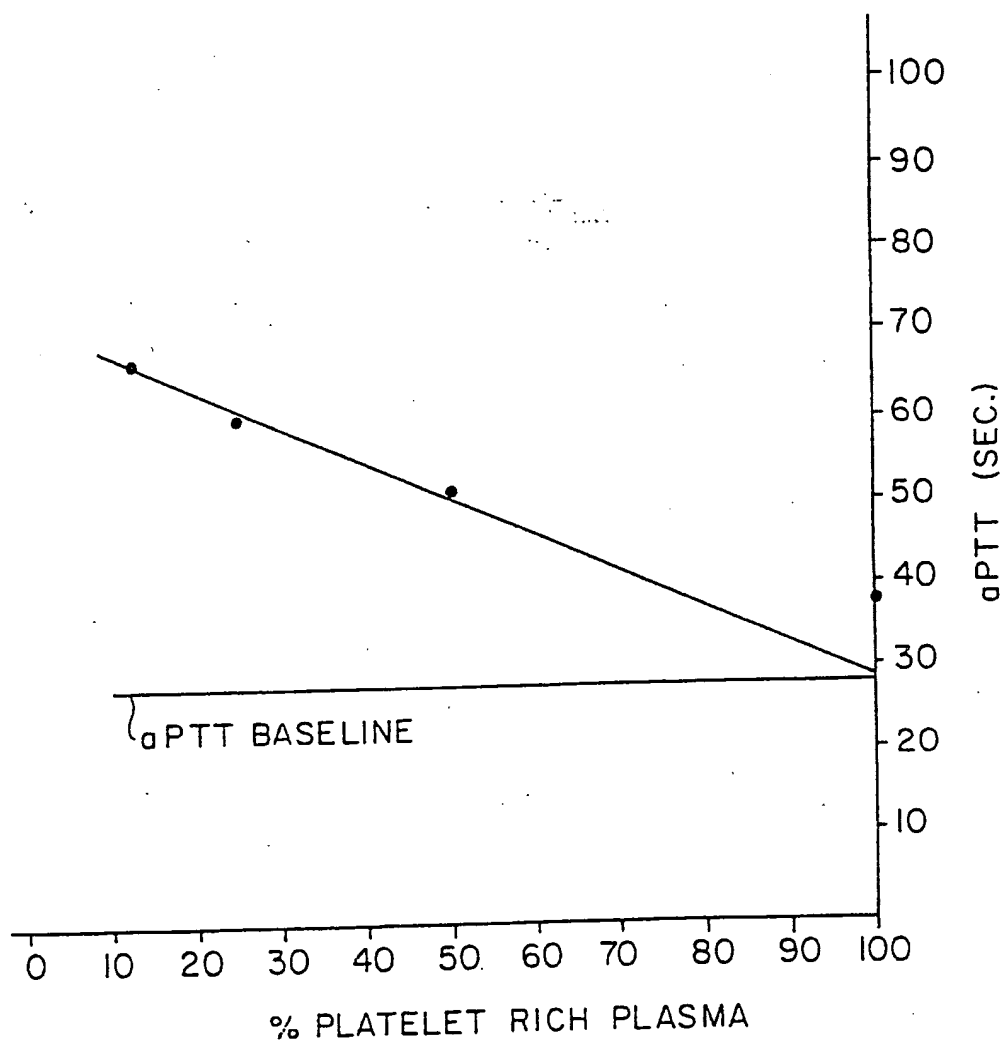


Fig. 3

PLATELET RESERVE FOR 50% DILUTION OF
PLATELET CONCENTRATE = 39 %

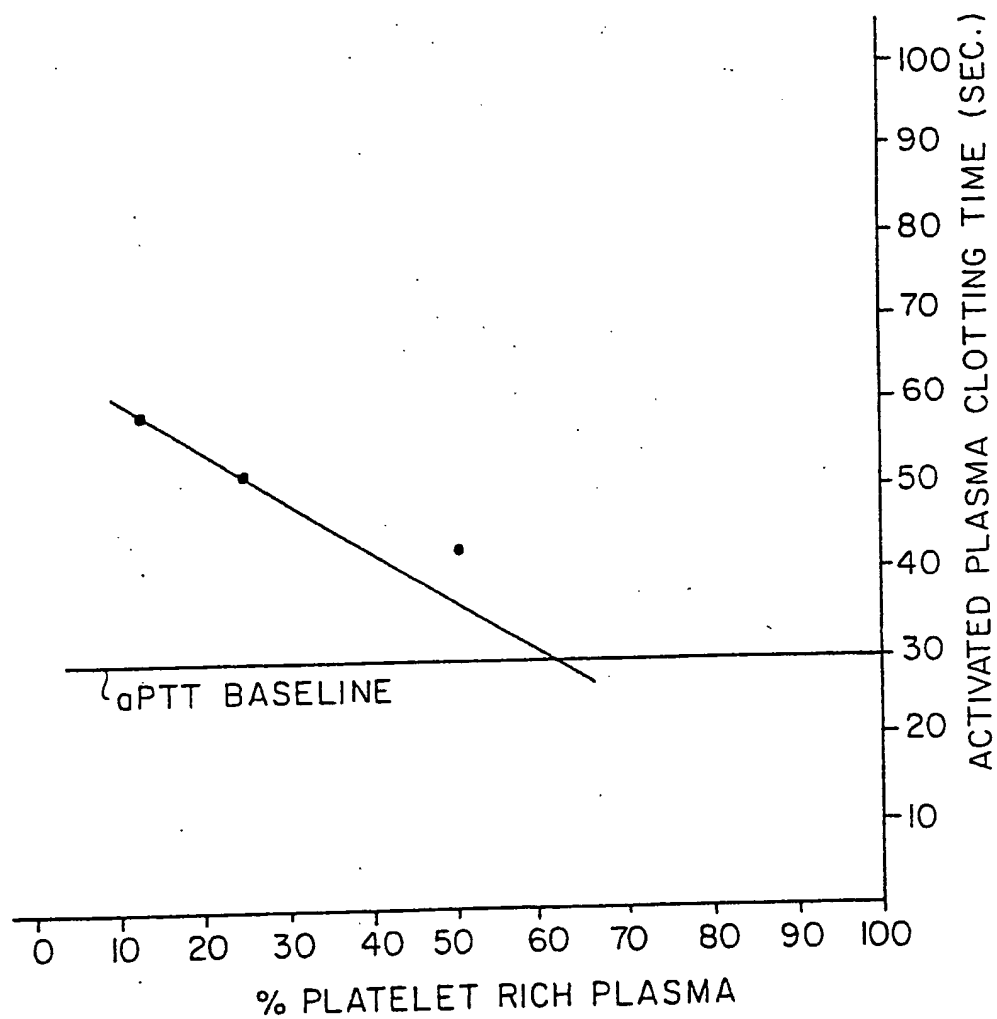


Fig. 4

PLATELET FACTOR 4 ASSAY

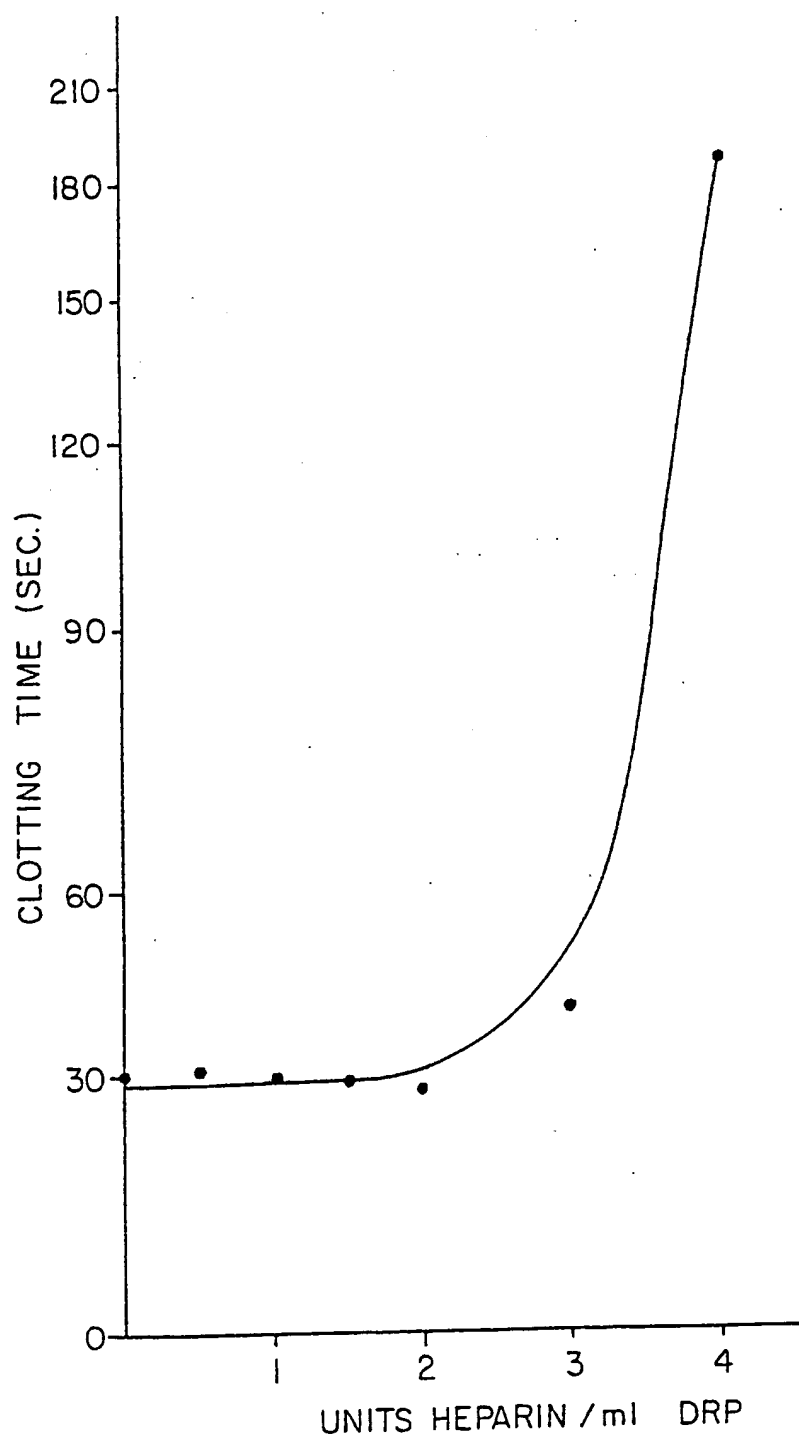


Fig.5

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/02580

| I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C12Q 1/56; G01N 33/86; A01N 59/20, 59/16 U.S.Cl.: 435/13; 436/69; 514/834; 424/630, 638, 646 | | | | | | | | | | | | | | | | | | | | |
|---|--|--------------------------------------|---|--|-------------------------------------|--|---|--------------------------------------|--------|---|----|---|---|--------------------------------------|---|---|----|--------|---|----------------------------------|
| II. FIELDS SEARCHED Minimum Documentation Searched * <table border="1"> <tr> <th>Classification System</th> <th>Classification Symbols</th> </tr> <tr> <td>U.S.Cl.</td> <td>435/13; 436/69; 514/834; 424/630, 638, 646</td> </tr> </table> Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched * APS, SEARCH TERMS: COAGULAT?, ASSAY, ELLAGIC, BLOOD | | | Classification System | Classification Symbols | U.S.Cl. | 435/13; 436/69; 514/834; 424/630, 638, 646 | | | | | | | | | | | | | | |
| Classification System | Classification Symbols | | | | | | | | | | | | | | | | | | | |
| U.S.Cl. | 435/13; 436/69; 514/834; 424/630, 638, 646 | | | | | | | | | | | | | | | | | | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT * <table border="1"> <thead> <tr> <th>Category *</th> <th>Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th>Relevant to Claim No. ¹³</th> </tr> </thead> <tbody> <tr> <td>Y</td> <td>M. Windholz. "The Merck Index" published 1976 by Merck + Co. (Rathway, NJ) see p.466.</td> <td>1-4, 15-17.41</td> </tr> <tr> <td>P, Y</td> <td>US, A, 4,985,354 (Toyomaki et al.) 15 January 1991. see column 3.</td> <td>29</td> </tr> <tr> <td>Y</td> <td>US, A, 4,455,377 (Finnerty et al.) 19 June 1984, see columns 1 and 2.</td> <td>1, 15, 18-19, 23-25.28-30, 33-36, 38</td> </tr> <tr> <td>Y</td> <td>US, A, 4,666,831 (Janoff et al.) 19 May 1987. see column 1 and 2.</td> <td>18</td> </tr> <tr> <td>X Y</td> <td>US, A, 3,486,981 (Speck) 30 December 1969. see entire document.</td> <td>18-19, 23 1-4.15-16.28, 33-34</td> </tr> </tbody> </table> | | | Category * | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ | Y | M. Windholz. "The Merck Index" published 1976 by Merck + Co. (Rathway, NJ) see p.466. | 1-4, 15-17.41 | P, Y | US, A, 4,985,354 (Toyomaki et al.) 15 January 1991. see column 3. | 29 | Y | US, A, 4,455,377 (Finnerty et al.) 19 June 1984, see columns 1 and 2. | 1, 15, 18-19, 23-25.28-30, 33-36, 38 | Y | US, A, 4,666,831 (Janoff et al.) 19 May 1987. see column 1 and 2. | 18 | X Y | US, A, 3,486,981 (Speck) 30 December 1969. see entire document. | 18-19, 23 1-4.15-16.28, 33-34 |
| Category * | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ | | | | | | | | | | | | | | | | | | |
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| Y | US, A, 4,455,377 (Finnerty et al.) 19 June 1984, see columns 1 and 2. | 1, 15, 18-19, 23-25.28-30, 33-36, 38 | | | | | | | | | | | | | | | | | | |
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| X Y | US, A, 3,486,981 (Speck) 30 December 1969. see entire document. | 18-19, 23 1-4.15-16.28, 33-34 | | | | | | | | | | | | | | | | | | |
| * Special categories of cited documents: ¹⁰ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "Z" document member of the same patent family | | | | | | | | | | | | | | | | | | | | |
| IV. CERTIFICATION <table border="1"> <tr> <td>Date of the Actual Completion of the International Search</td> <td>Date of Mailing of the International Search Report</td> </tr> <tr> <td>02 July 1991</td> <td>29 JUL 1991</td> </tr> <tr> <td>International Searching Authority</td> <td>Timothy J. Reardon <i>TJ Reardon</i></td> </tr> <tr> <td>ISA/US</td> <td></td> </tr> </table> | | | Date of the Actual Completion of the International Search | Date of Mailing of the International Search Report | 02 July 1991 | 29 JUL 1991 | International Searching Authority | Timothy J. Reardon <i>TJ Reardon</i> | ISA/US | | | | | | | | | | | |
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| International Searching Authority | Timothy J. Reardon <i>TJ Reardon</i> | | | | | | | | | | | | | | | | | | | |
| ISA/US | | | | | | | | | | | | | | | | | | | | |

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☒ Claim numbers 17, 18, 23-24, 28-29, 33, 41, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

Use of language such as "hydroxy-substituted aromatic compound" renders these claims so indefinite as to result in no meaningful search capable of being carried out.

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

(See attachment A)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone practice

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort proportional to a additional fee, the International Searching Authority did not make payment of any additional fee.

Remarks on Protest:

☐ The additional search fees were accompanied by a protest and a statement.

☐ The protest was accompanied by a statement and a statement of the facts.

Attachment to PCT Telephone Memorandum

OBSERVATION WHERE UNITY OF INVENTION IS LACKING

Reasons for holding lack of unity of invention

PCT rules 13.1 and 13.2 allow the grouping together as a "single general inventive concept" the following: a First (appearing) Product, a First (appearing) Process of making the First Product and a First (appearing) Method of using the First Product. Group I meets this description.

Further methods of using or making the First Product are considered additional inventions. Groups VII-VIII are drawn to distinct methods of using the First product.

Products different from the First Product and methods of using or making these additional products are considered separate inventions. Groups II-VI are distinct because they are concerned with products that differ in composition from the First Product.

Attachment to PCT Telephone Memorandum

OBSERVATION WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

- I. Claims 1-16, 33-35 are drawn to a first product, containing ellagic acid, for activating coagulation. This group also includes a first method of making the first product and a first method of using the first product. Class 435 subclass 13.
- II. Claim 17 are drawn to a second product, containing gallatic acid or tannin, for activating coagulation. Class 435, subclass 13.
- III. Claims 18-27 are drawn to a third product for activating coagulation, containing platelet substitute. Class 435, subclass 13.
- IV. Claim 28 are drawn to a first method for detecting lupus anticoagulant, using the third product. Class 436, subclass 69.
- V. Claims 29-32 are drawn to a fourth product, containing dextran sulfate. Class 435, subclass 13.
- VI. Claims 33-34 are drawn to a first method for assaying coagulation, using the second product. Class 435, subclass 13.
- VII. Claims 33, 36-40 are drawn to a second method using the first product, for assaying platelet-rich or platelet-poor plasma. Class 435, subclass 13.
- VIII. Claim 41 are drawn to a third method of using the first product, for stopping bleeding. Class 435, subclass 2.

ATTACHMENT A (CONTINUATION OF PART VI)

VI. OBSERVATION WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

- I. Claims 1-16, 33-35 are drawn to a first product, containing ellagic acid, for activating coagulation. This group also includes a first method of making the first product and a first method of using the first product. Class 435 subclass 13.
- II. Claim 17 are drawn to a second product, containing gallatic acid or tannin, for activating coagulation. Class 435, subclass 13.
- III. Claims 18-27 are drawn to a third product for activating coagulation, containing platelet substitute. Class 435, subclass 13.
- IV. Claim 28 are drawn to a first method for detecting lupus anticoagulant, using the third product. Class 436, subclass 69.
- V. Claims 29-32 are drawn to a fourth product, containing dextran sulfate. Class 435, subclass 13.
- VI. Claims 33-34 are drawn to a first method for assaying coagulation, using the second product. Class 435, subclass 13.
- VII. Claims 33, 36-40 are drawn to a second method using the first product, for assaying platelet-rich or platelet-poor plasma. Class 435, subclass 13.
- VIII. Claim 41 are drawn to a third method of using the first product, for stopping bleeding. Class 435, subclass 2.

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